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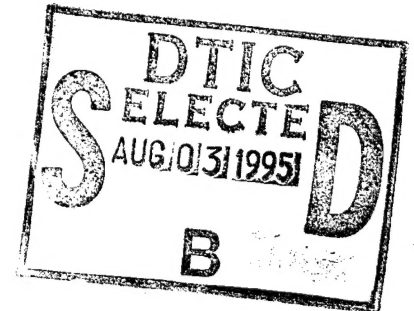
TITLE: Triplex Forming Therapeutic Agents for Breast Cancer

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13. ABSTRACT (Maximum 200 words)  The overall goal of this grant is to develop sequence specific DNA binding compounds which are targeted by triplex DNA formation to the promoters of the c-myc, neu, and c-Ha-ras genes. These genes play important roles in the malignant phenotype of breast carcinoma cells. During the past year, we have demonstrated that the major problem with the treatment of tumors in intact animals with TFOs is delivery of intact oligonucleotides to the nuclei of tumor cells. We have attempted to circumvent this problem by the creation of plasmids which express triplex forming transcripts. These plasmids appear to have significant antiproliferative characteristics. We have also cloned and sequenced a set of cDNAs complementary to RNA molecules which appear to regulate c-myc expression via triplex formation. We have also demonstrated that the nontranslated tumor suppressor gene, H19, downregulates expression of the c-myc promoter. This gene also contains a sequence which is highly homologous to the triples forming region of the c-myc P1 promoter. We think that the discovery and characterization of triplex forming transcripts promises to lead to effective transcriptional <u>inhibitory transcripts</u> with potential as gene therapy vectors.				
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## PROGRESS REPORT: DOD BREAST CANCER GRANT

Donald M. Miller, M.D., Ph.D.

During the past year our work has concentrated on the development of triplex based gene therapeutic approaches. During that time we have developed expression vectors which express putative triplex forming transcripts and appear to be antiproliferative. We have also developed evidence that the nontranslated tumor suppressor gene H19 functions by triplex formation with the c-myc promoter. We have also identified physiologic triplex forming transcripts and have cloned cDNAs for these genes. The identification of putative triplex forming transcripts has suggested that these sequences may provide considerable advantage as gene therapy agents. We have continued to be quite productive with seven publications during the past year, with six manuscripts currently in press and eight under review at the present time. In addition, the work supported by this grant has resulted in four invited book chapters or reviews during the past year.

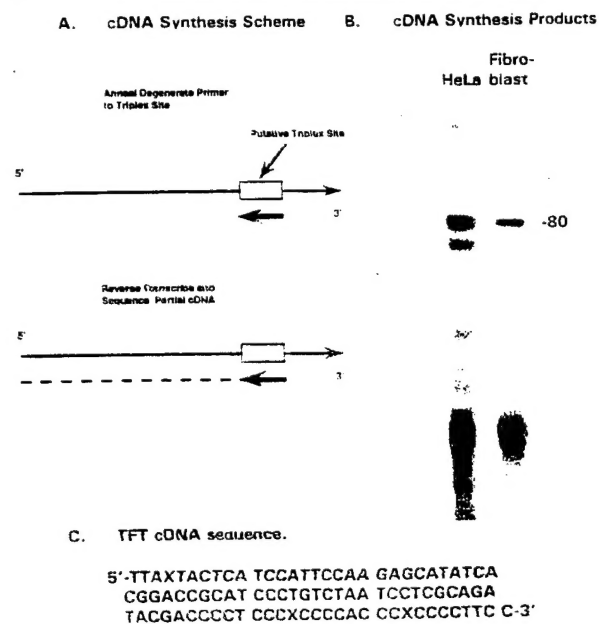
### 1. The identification and cloning of physiologic c-myc triplex forming transcripts (Manuscript in Preparation).

Our interest in the potential biologic role of triplex formation was stimulated by the fact that triplex forming sequences are generally located in important regulatory regions of growth control genes. This observation, along with the fact that triplex binding proteins have been identified, and that antitriplex antibodies react specifically with human chromatin, suggested that triplex formation might be a physiologic regulatory mechanism. We and others have performed affinity hybridization experiments in an effort to detect physiologic triplex forming RNA molecules. These experiments have been negative. However, we developed a new hybridization approach which utilizes the higher affinity of duplex DNA hybridization to identify putative triplex forming transcripts. This "duplex capture" technique uses a biotin:single stranded oligonucleotide affinity column to capture potential triplex forming molecules by duplex hybridization. Our preliminary data indicates that triplex forming RNA molecules targeted to the c-myc promoter do exist in various tumor cell lines in relatively low concentrations. The existence of these molecules was confirmed by "triplex blot" experiments in which radiolabelled triplex forming double stranded oligonucleotides were hybridized to blotted RNA. It is clear that the c-myc triplex forming sequence binds to an RNA band which corresponds to the band which hybridizes to the complementary oligonucleotide to the putative triplex forming transcript.

In order to further characterize the putative triplex forming transcript, we first enriched for low molecular weight RNA with lithium chloride precipitation. We then used the hybridized oligonucleotide to initiate cDNA synthesis. This allowed us to identify a single prominent band (**Figure 1**) which represented a partial cDNA molecule of 89 bp. This molecule was purified and sequenced, as shown. The sequence of this cDNA was identical from two cell lines (HeLa and fibroblasts). Using this sequence we have generated a 5' PCR primer for amplification. After enriching for low molecular weight RNA, we ligated a 21 bp oligonucleotide to the 3' end of all RNA species. Using the complement of the ligated 21 bp oligonucleotide, we synthesized a cDNA with reverse transcriptase. Successful RNA ligation was determined by gel analysis which yielded cDNAs corresponding to low molecular weight species up to approximately 1 kb.

For PCR amplification of specific products, we used the 5' gene-specific primer and the 21 bp primer. This has generated several PCR products ranging in size from 60 to 120 bp (**Figure 2**). More stringent PCR conditions have resulted in a single predominant PCR product of 120 bp. We have recently cloned this amplified cDNA, which we have named MBT-1 (myc binding transcript). We have generated complete sequence data for this 120 base transcript.

Preliminary expression data for this transcript is quite interesting. Northern hybridization of breast



**Figure 1.** TFT cDNA synthesis.

carcinoma cell lines with an oligonucleotide complementary to the putative c-myc TFT hybridizes to two bands at 2.3 kb and approximately 200 bp. The point should be made that this oligonucleotide will not hybridize to c-myc mRNA or any known coding sequence, although it may hybridize to the transcript of the H19 gene.

2. The nontranslated tumor suppressor H19 downregulates transcriptional activity of the c-myc promoter and may encode a triplex forming RNA (Manuscript in Preparation).

We have identified a sequence in the H19 coding sequence which is a near match with the triplex forming sequence in the c-myc PI promoter. We have performed cotransfection experiments with the c-myc promoter/luciferase plasmid and have shown that expression of H19 mRNA inhibits c-myc promoter activity. In addition, we have subcloned 250 bp of the H19 gene which contains the putative triplex forming sequence and we have shown that expression of this sequence also results in downregulation of the c-myc promoter (Figure 3).

Although there are several potential mechanisms by which the H19 transcript could downregulate the c-myc promoter, we think that triplex formation is the most likely, since the putative triplex forming sequence is an excellent match and the 250 bp insert of H19 is equally effective.

3. Triplex formation by the cyclin D1 promoter results in inhibits promoter function in vivo (Manuscript Submitted).

Cyclin D1 is an excellent target for gene-specific transcriptional inhibition. The promoter of the human cyclin D1 gene contains an 18 bp purine-pyrimidine rich sequence located from -116 to -99 from the transcription start site. This sequence, which is a binding site for Sp1, is not purely polypurine/polypyrimidine but contains three G:C interruptions. A potential triplex forming oligonucleotide targeted to the human cyclin D1 pur:pyr motif was designed in parallel and antiparallel orientation with respect to the purine rich strand, containing guanine to recognize GC (G \*GC triplets) and thymine to recognize CG (T\*CG triplets). The parallel oligonucleotide was used as a control oligonucleotide which had the same base composition, but would not form triplex DNA.

Triplex formation was demonstrated by gel mobility shift analysis and DNase I footprint analysis. The addition of increasing amounts of antiparallel oligonucleotide, CD18ap, relative to double stranded target sequence, results in the gradual shift from duplex to a distinct triplex band, while the parallel, nontriplex forming oligonucleotide does not. This indicates that triplex formation occurs with relatively high affinity and is specific for the antiparallel oligonucleotide. The sequence specificity of this interaction was demonstrated by DNase I footprint analysis. This set of experiments demonstrated that the TFO interacts very specifically with its target sequence, while the parallel, nontriplex forming oligonucleotide, does not.

In order to optimize the conditions for intracellular transcriptional inhibition of cyclin D1, we have

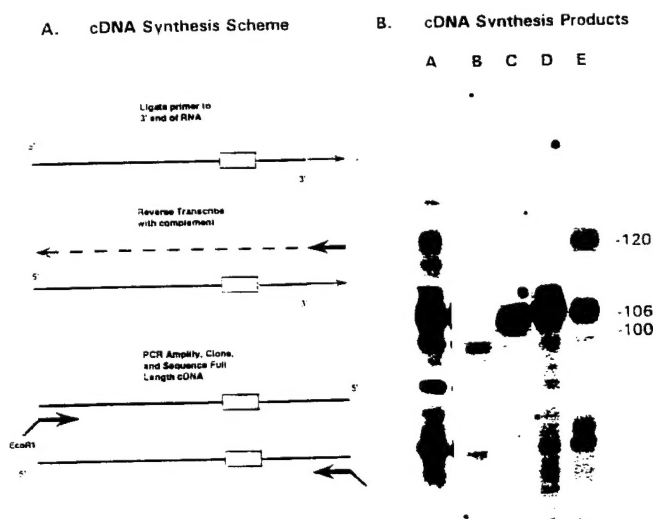


Figure 2. Synthesis and Cloning of Full Length TFT cDNA.

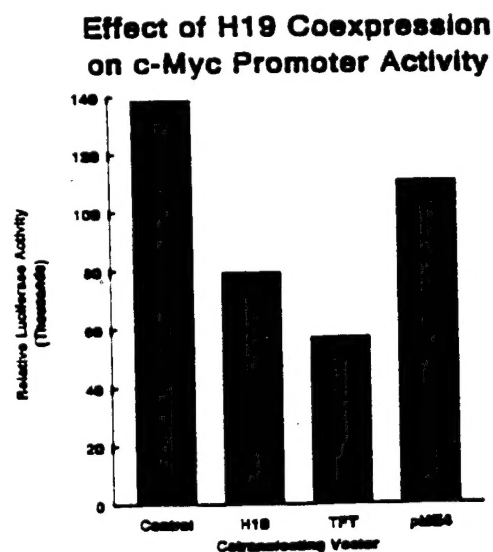


Figure 3. Inhibition of c-myc Promoter Expression by H19 Cotransfection.



stably transfected HeLa cells with a vector containing the luciferase gene driven by the cyclin D1 promoter. These cells express relatively high levels of luciferase activity. When the transfected cells are treated with 4  $\mu$ m phosphorothioate triplex forming oligonucleotide for 48 hours after electroporation, there is 60-80% inhibition of luciferase activity, while the parallel oligonucleotide does not affect luciferase expression (**Figure 4**). Conversely, stably transfected cells containing the luciferase gene in an identical vector, but under the control of the CMV promoter do not demonstrate inhibition after treatment with the cyclin D1 targeted TFO.

4. The identification of a novel P2 c-myc triplex forming sequence in the ME1a1 regulatory region of human and murine c-myc 5'-flanking region (Biochemistry, in press).

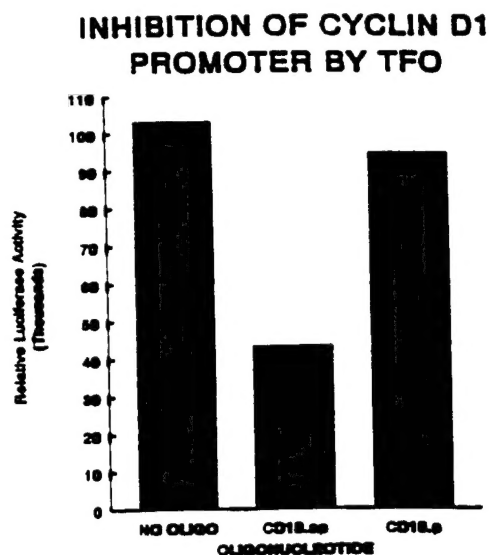
The P2 promoter is the dominant transcription start site of the human and murine c-myc gene under almost all circumstances. Our data, and that of Hogan, indicated that the P1 targeted TFO only inhibits in vitro transcription of the P1 promoter. Therefore, we were interested in the identification of a potential triplex forming region in the P2 promoter. Since the submission of the original proposal we have shown that the ME1a1 binding site for the Maz positive regulatory protein is capable of triplex formation. Maz is a strong positive regulator of c-myc expression. Triplex formation by its binding site in the human c-myc promoter prevents protein binding at relatively low TFO concentrations (ratios of 10:1 or greater, depending on reaction conditions). In vitro transcription of the c-myc P2 promoter is inhibited by the P2 targeted TFO. Experiments to determine the effect of phosphorothioate TFOs targeted to the P2 promoter are currently underway. The identification of the P2 promoter triplex forming sequence is an extremely important observation, because we can now target either P1 or P2, or both, in our animal experiments.

6. Triplex formation by the murine c-myc P1 and P2 promoter (Biochemistry, in press; appended).

Although potential triplex forming sequences have been identified in the promoters of a number of genes (see above), all of the published work with triplex formation by biologically significant sequences has used sequences of human promoters. In order to characterize the potential toxic effects of triplex forming compounds, it will be necessary to identify triplex forming sequences in murine promoters. We have identified triplex forming sequences in the murine P1 and P2 promoters, homologous those in the human c-myc gene. These sequences are not cross reactive with the human sequence (the human triplex forming oligonucleotide does not form triplex with the murine sequence or vice versa.) The murine P1 sequence binds both the murine and human Puf (Nm23) protein and is in a region which is an important regulatory region. Recent studies in our laboratory have demonstrated that triplex formation by this region prevents regulatory protein binding and inhibits in vitro transcription. The identification of a murine triplex forming sequence allows the design and execution of novel toxicity studies which were not previously feasible.

7. Construction of c-myc and cyclin D1 /luciferase "indicator" constructs to measure transcriptional inhibition (manuscript submitted).

As noted above, we have constructed marker gene vectors in which the c-myc and N-ras promoters are cloned upstream of the luciferase gene. We have stably transfected HeLa cells with the cyclin D1 vector (described above) and have recently stably transfected YC8 cells with the human and murine c-myc/luc vectors. Interestingly, while the majority of the cyclin D1 transfected cells demonstrate inhibition with the cyclin D1 TFO, a subset do not. This suggests that the site of integration and the integrity of the integrated promoter may affect the mechanism of regulation of the luciferase gene under these conditions. The c-myc promoter vector has already been utilized to demonstrate that the expression of the H19 transcript inhibits



**Figure 4. Inhibition of Cyclin D1 Promoter Activity by Cyclin D1 Targeted TFO.**

c-myc promoter function. The stably transfected YC8 cell lines will be critically important for the animal studies outlined in this proposal. They will allow us to determine oligonucleotide delivery and function simultaneously and will provide an excellent model to compare in vitro and in vivo systems.

8. Development of a novel single cell assay for the detection of intracellular oligonucleotides (Manuscript Submitted).

In order to reach its target sequence in intact cells a TFO must traverse the cell membrane, endosomes and nuclear membrane. It has been difficult, in the past, to determine the extent to which oligonucleotides reached the nucleus. We have addressed that problem with the development of a novel assay which measures cell to cell and cell line to cell line differences in oligonucleotide uptake. In order to detect TFO we synthesized triplex forming oligonucleotides with a "tail" of several bromodeoxyuridine (BrdU) nucleotides. These oligonucleotides can be visualized in intact cells by an anti-BrdU antibody using standard immunohistochemistry methods. Five cell lines were treated with BrdU modified TFO delivered by adenovirus/polylysine complexes, liposomes, or incubation with free phosphorothioate TFO. The approximate per centage of cells taking up oligonucleotide, as well as the relative intensity of staining (+ to +++) were quantitated.

TABLE 1

Delivery System	SK-BR-3	SK-OV-3	HeLa	SK-MEL-28	G401
Ad/Pl	75%(+++)	90%(+++)	50%(++)	100%(+++)	50%(++)
Liposome TFO	40%(++)	90%(++)	40%(+)	40%(+)	30%(+)
Free TFO	10%(++)	40%(+)	30%(+)	90%(+)	5%(+)

It is clear that the uptake of free TFO varies widely between cell lines, and some cell lines take up minimal amounts of oligonucleotide. In contrast, as noted above, Ad/pl complexes seem capable of delivering high quantities of TFO to all cell types. This assay system provides a convenient means of optimizing delivery of oligonucleotides to specific cell types. We have also shown that this method can detect oligonucleotides in tumors in intact animals, which is of obvious importance to this application.

9. Adenovirus assisted delivery of triplex forming oligonucleotides (Manuscript in preparation).

In an attempt to enhance delivery of the triplex forming oligonucleotide targeted to the neu gene, we have applied the gene transfer technology now in use for therapeutic genetic manipulations. Specifically, we have formed polylysine-oligonucleotide complexes coupled to a replication defective adenovirus by means of a non-neutralizing anti-adenovirus monoclonal antibody-polylysine conjugate. This complex takes advantage of the receptor mediated endocytosis mechanism by which the adenovirus infects cells, as well as utilizing the endosomolytic ability of the viral capsid. We have utilized this method to deliver triplex forming oligonucleotides to two breast cancer cell lines, MCF-7 and SkBR-3. We have demonstrated high efficiency DNA transfer to the nucleus, as measured by a luciferase reporter gene. We have documented luciferase expression of  $5 \times 10^5$  light units in  $10^7$  breast cancer cells with this delivery system at 16-48 hours. We have found adenocomplexes with a combination of luciferase reporter plasmid and triplex forming oligonucleotide at a 1:1 ratio and achieved similarly high levels of luciferase activity in both cell lines. Radiolabelling experiments to document the delivery of oligonucleotide and its biologic effects are currently underway. The preliminary results demonstrate that transfer of triplex forming oligonucleotides to the nucleus is possible using this system.

10. Triplex forming oligonucleotides and minor-groove DNA binding drugs have synergistic effects (Manuscript Submitted).

We have shown, gel shift experiments, that mithramycin binding prevents triplex formation. However, this coinubation of the target sequence with both triplex forming oligonucleotide and mithramycin results in a dramatic increase in the ability of each modality to inhibit protein binding to the target sequence. In other words, the addition of mithramycin at a concentration of  $10^{-5}$  M results in a 10 fold reduction in the TFO concentration required to completely inhibit protein binding to the target sequence. Likewise, the presence of TFO substantially increases the ability of mithramycin to inhibit protein binding. This result

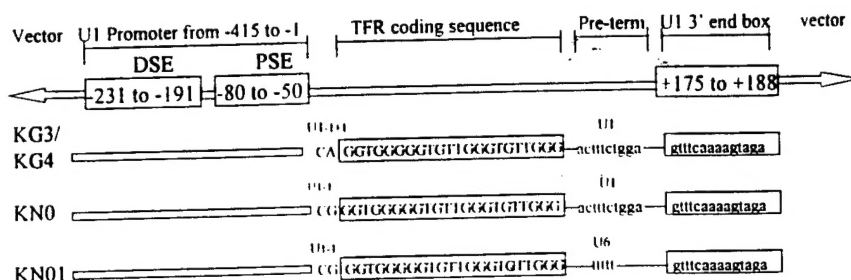


suggests that simultaneous treatment with DNA binding drugs and triplex forming oligonucleotides may have very synergistic antiproliferative effects.

#### 11. Construction of TFT expressing vectors.

We have constructed vectors which are engineered to express triplex forming transcripts targeted to the c-myc and c-Ha-ras promoter sequences. These vectors will be utilized to test potential gene therapy approaches in animal model systems. As shown in **Figure 5**, these vectors take advantage of the Pol III promoter and are specifically designed to express small transcripts. These vectors have been shown to express the expected transcript in transfected cells. Experiments designed to test the effect

of the expressed TFTs on expression of endogenous cmVc and a myc/luciferase target gene vector are currently underway. These vectors are of obvious importance in the experiments which we are proposing.



**Figure 5. TFT Expression Vectors.**

#### 12. Summary.

The studies performed since the submission of the previous progress report have provided considerable additional impetus for the continued development of c-myc targeted DNA binding compounds. We have developed several animal models for the in vivo testing and toxicity analysis of these compounds, and we have used these systems in preliminary experiments to demonstrate that these compounds are antiproliferative in vivo. The development of stably transfected cell lines containing the luciferase gene driven by target promoters provides an excellent means of comparing in vitro and in vivo transcriptional inhibition. Our novel single cell oligonucleotide assay system will allow us to characterize delivery and function of triplex forming oligonucleotides in both tissue culture and whole animal model systems. In addition, the cloning and sequencing of cDNAs which are complementary to putative triplex forming transcripts promises to provide new insight into the design of optimal triplex based transcriptional inhibitors.

#### PUBLICATIONS SINCE LAST REVIEW:

##### Peer Reviewed Publications:

1. Charles Mayfield, Mark Squibb, Donald Miller: Inhibition of Nuclear Protein Binding to the Human Ki-ras Promoter by Triplex-Forming Oligonucleotides. *Biochemistry*, 33:3358-3363, 1994.
2. Mayfield, C. and Miller D.M.: Effect of abasic linker substitution on triplex formation, Sp1 binding, and specificity in an oligonucleotide targeted to the human Ha-ras promoter. *Nuc. Acids Res.* 22:1909-1916, 1994.
3. C. Mayfield S. Ebbinghaus, J. Gee, D. Jones, B. Rodu, M. Squibb and D. Miller: Triplex formation by the human Ha-ras Promoter Inhibits Sp1 Binding and in Vitro Transcription. *J. Biol. Chem.*, 27:18232-1823, 1994.
4. Chaudhary, D., Miller, D.M.: The c-myc promoter binding protein MBP-1 prevents TBP binding to the TATA box sequence. *Biochemistry*, 34:3438-3445, 1995.
5. Ray, R., Shrestha, K., and Miller, D.M.: Isolation and characterization of a cDNA clone showing c-myc binding activity. *Int. J. Cancer*, 5:111-115, 1994.
6. Chen, S.J., Chen, Y.F., Miller, D.M., Oparil, S.: Mithramycin Inhibits Myointimal Proliferation after Balloon Injury of the Rat Carotid Artery In Vivo. *Circulation*, 90:2468-2473, 1994.
7. Campbell, V.W., Roesel, J., Rigsby, D., Sanchez, J.D., Ray, R., Shrestha, K., Nalluswami, K., Stone, T., Miller, D.M.: The G-C Specific DNA Binding Drug, Mithramycin, Selectively Inhibits Transcription of the c-MYC and c-HA-RAS Genes in Regenerating Liver *Am. J. Med. Sci.*, 307:167-172, 1994.

8. Ray, RB, Fontana, J, Miller, DM: Human breast carcinoma cells show correlation in expression of c-myc oncogene and the c-myc promoter binding protein, MBP-1. *Int. J. Cancer*, In Press.
9. Jones, D.E., Cui, D.M., Miller, D.M.: Expression of  $\beta$ -Galactosidase Under the Control of the Human c-Myc Promoter in Transgenic Mice. *Oncogene*, in press.
10. Tran-Patterson R, Davin D, Miller DM: Epidermal growth factor Secreted from the salivary gland is necessary for liver regeneration. *Amer. J. Phys.*, in press.
11. Sanders, G.S., Mayfield, C.A., Kim, H.G., Ebbinghaus, S.W., Rodu B., Squibb, M., Miller D.M.: Time dependence of triplex formation by a novel HIV-1 targeted triplex forming oligonucleotide and its 3' acridine conjugate. *Biochemistry*, in press.
12. Reddoch, J., Miller, D.M.: The Nm23 Binding Site in the Murine c-Myc Promoter which Forms Triplex DNA. *Biochemistry*, In press.
13. Kim, H., Mayfield, C., Sanders, G., Ebbinghaus, S., Vigneswaran, N., Miller, R., Miller, D.M.: Triplex formation by the Me1A1 binding site of the human c-myc promoter. *Biochemistry*, In Press.

#### **Manuscripts submitted:**

14. Ebbinghaus, S., Vigneswaran, N., D., Rodu, B, Miller, D.M.: Heterogeneous uptake of triplex forming oligonucleotides by tumor cells in vitro. Submitted to *Cancer Research*.
15. Kim, H., Mayfield, C., Sanders, G., Ebbinghaus, S., Vigneswaran, N., Miller, R., Miller, D.M.: Triplex formation by the human cyclin D1 promoter. Submitted to *J. Biol. Chem*.
16. Blume SW, Gee JE, Shrestha K, Ray R, Snyder RC, and Miller DM; Triple Helical versus Tetrahelical Assembly: Differential Effects of Divalent Transition Metal Cations on Competing Non-Watson Crick Intermolecular Interactions. Submitted to *J. Mol. Biol*.
17. Miller, D.M., Thomas, S.D., Snyder, R., Blume, S., Ray, R., Sanchez, J., Krauss, R., Harris, M., Koller, C.A.: The DNA binding drug, mithramycin, inhibits transcription of the HIV LTR in vitro. Submitted to *AIDS Research*.
18. Vigneswaran, N., D., Rodu, B, Miller, D.M.: Triplex DNA Formation is Prevented by Minor Groove Binding Drugs. Submitted.
19. Grizzle, W.E., Perucho, M., Myers, R.B., Miller, D.M., Hubbard, W.J., Srivastava, S.: Prostatic Adenocarcinoma: Involvement of Suppressor Genes. Submitted.
20. Miller, D.M., Chen, S.J., Chen, Y.F., Oparil, S., Lambert C.M.: Mithramycin Inhibits Myointimal Proliferation after Balloon Injury of the Pig Coronary Artery In Vivo. Submitted to *Circulation Research*.
21. Rodu, B., James, R., Garciello, V.T., Hubbard, B., Jones, D.E., Miller, D.M.: Cloning and characterization of genes which encode small regulatory RNA molecules. In preparation.

#### **Book Chapters and Review Articles:**

22. J.E. Gee, Ebbinghaus, C. Mayfield, G. Sanders, B. Shrestha, B. Rodu, K. Rabenau, D.M. Miller.: Potential therapeutic usefulness of intermolecular triplex DNA in Cancer Therapy in the Twenty-First Century; *Molecular and Immunologic Approaches*; Huber, B. and Carr, B.I, Ed.; Futura; Mount Kisco, N.Y. pp. 163-177, 1994.
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25. Mayfield C., Ebbinghaus S., Sanders G., Kim H., Vigneswaran N., Rodu B., Jones D., Miller D.M.: Animal Models for Antigen Therapy. *Transgenic Research*, in press.

#### **Abstracts:**

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2. J.P. Broome and D.M. Miller.: Attitudes of Sickle Cell Anemia Patients towards their medical care givers. *Clin. Res.*, 42:228A, 1994.

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